The problem of bacterial contamination in studies of protein synthesis by isolated mitochondria

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There has been some question concerning the role of contaminating bacteria in observations of protein synthesis in isolated mitochondria. Although Roodyn, Reis, and Work (1) found that preparations of rat liver mitochondria do contain bacteria, Roodyn, Freeman, and Tata (2) later concluded that the bulk of amino acid incorporated was introduced into mitochondrial protein. On the other hand, Sandell et al. (3) recently reported that no incorporation could be observed in sterile, filtered mitochondrial systems.

This communication reports a highly efficient means of separating labeled mitochondrial membrane proteins from contaminating bacteria. The results obtained establish conclusively that isolated mitochondria possess intrinsic capacity for protein synthesis and permit definition of conditions for studying this process unambiguously. However, they also show that significant amino acid incorporation may occur into contaminating bacteria, that bacteria may compete for and "monopolize" labeled amino acids, and that bacterial contamination may account for two properties previously attributed to protein synthesis in mitochondria, viz. insensitivity to ribonuclease (1, 4, 5) and non-requirement of ATP (6).

Methods. Rat liver mitochondria were prepared by the method of Schneider (7) and washed three times in sucrose. For treatment with ribonuclease, the mitochondria were suspended in 100 mM KCl - 50 mM Tris chloride, pH 7.5, at 2.5 mg protein per ml and crystalline bovine pancreatic ribonuclease was added at 0.2 mg per ml. After 20 min at room temperature, the mitochondria were recovered by centrifugation, re-suspended in 0.25 M sucrose, and added to the test medium. In control experiments, ribonuclease was omitted. All incubations were carried out in a Dubnoff shaker at 37°, using open beakers. The acid-insoluble protein fraction was reprecipitated, washed, extracted with ethanol-ether, plated, and counted as described elsewhere (8).

Results. Data in Table 1 show that treatment of mitochondria with sodium deoxycholate at 10 mg per g protein, which is just sufficient to cause complete lysis, resulted in solubilization of 75% of the mitochondrial protein. About 80% of the mitochondrial protein that failed to dissolve sedimented in 0.5 M sucrose but floated in 1.5 M sucrose; this fraction was rich in both phospholipid and RNA and is designated the membrane fraction (cf. 9). The remaining 20% of the deoxycholate-insoluble mitochondrial protein sedimented in 1.5 M sucrose (designated sediment fraction). The latter consisted chiefly of unlysed mitochondria and contained contaminating bacteria, since, in the absence of detergent, both intact mitochondria and bacteria sedimented in 1.5 M sucrose. No label appeared in the membrane fraction when labeled bacterial cells were fractionated. When mitochondria labeled with ^{14}C-leucine in a preceding incubation were submitted to the above lysis and fractionation procedure, the membrane fraction usually showed the highest specific radioactivity (Table 2). The addition of 10^5 cells of a mixture of S. typhimurium and M. lysodeikticus per ml (corresponding to the upper limit of bacterial contamination found by Roodyn et al. (2)) produced a highly radioactive sediment fraction but also caused lowering of the specific activity of the membrane fraction, probably due to monopoly of the available ^{14}C-leucine by the bacteria. In this case approximately half the protein synthesized was due to bacteria and half to mitochondria. When 10^6 bacterial cells per ml were added (two orders of magnitude less than required to produce a visible turbidity), the bacteria accounted for approximately 90% of the incorporation of amino acid in the complete system.

Table 1

Protein fractions obtained from deoxycholate-lysed mitochondria

Mitochondria (= 1 g protein) were suspended in 10 ml 250 mM sucrose-50 mM KCl-5 mM $MgCl_2$-50 mM Tris HCl, pH 7.6, and homogenized with 1.0 ml 10% sodium deoxycholate, pH 8.0. The suspension was transferred in four equal portions to tubes containing 3.0 ml 1.5 M sucrose and 3.0 ml 0.5 M sucrose, layered so as to form a discontinuous gradient, with the lysed mitochondria at the top. The tubes were centrifuged for 60 min at 40,000 rpm, yielding a clear yellow layer at the top ("soluble fraction"), a dense brown layer at the surface of the 1.5 M sucrose ("membrane fraction"), and an opaque pellet at the bottom of the tubes ("sediment fraction").

Fraction	% Total Protein	RNA (μmoles P/mg protein)	Phospholipid (μmoles P/mg protein)
Mitochondria	100	0.056	0.20
Soluble fraction	76	0.02	0.13
Membrane fraction	18	0.16	0.43
Sediment fraction	5.5	0.06	0.12

Table 2

Effect of added bacteria on the distribution of radioactivity in mitochondrial protein fractions

Incubation mixtures contained mitochondria (3.0 mg protein per ml), sucrose (100 mM), KCl (100 mM), KH_2PO_4 (10 mM), succinate (10 mM), $MgCl_2$ (10 mM), proline (5 mM), ATP (2 mM), DL-1-^{14}C-leucine (0.33 μC per ml; total leucine in system ~0.05 mM) and Tris buffer (33 mM), to give pH = 7.5 in a final vol. of 15 ml. After incubation for 30 min., 2.0 ml incubation mixture was added directly to trichloroacetic acid. The remainder was cooled to 0° and the mitochondria recovered by centrifuging 10 min. at 10,000 x g. The pellets were suspended in 1.0 ml 250 mM sucrose - 50 mM KCl - 5 mM $MgCl_2$ - 50 mM Tris HCl, pH 7.6, and homogenized with 0.05 ml 10% sodium deoxycholate, pH 8.0. The suspensions were then subjected to density gradient centrifugation (Table 1).

The bacteria added consisted of equal numbers of Salmonella typhimurium and Micrococcus lysodeikticus from log phase cultures, diluted in 0.05 M Tris chloride, pH 7.5.

Bacteria added cells/ml	Specific Radioactivity, as c.p.m./mg protein (figure in parenthesis = % total radioactivity)			
	Mitochondria	Soluble Fraction	Membrane Fraction	Sediment Fraction
None	14.5	9.3(39)	41.0(46)	16.5(16)
10^5	25.0	9.2(31)	10.1(7)	167 (62)
10^6	103	7.6(5)	15.3(2)	1,038 (93)

In this series of experiments the same precautions were taken throughout to minimize external sources of bacteria (fresh reagents were prepared for each experiment, including the sucrose used for homogenization.) Even without added bacteria some incorporation always took place in the sediment fraction.[1] This was usually less than 20% of the total radioactivity; but the specific radioactivity varied according to the amount of unlysed mitochondria remaining after detergent treatment.

Further experiments compared the effect of inhibitors on amino acid incorporation into the membrane fraction and into the sediment fraction containing bacteria (Table 3). Labeling in both fractions was inhibited by chloramphenicol, which is known to inhibit protein synthesis in bacteria and in mitochondria, but not in microsomes (10, 11). When ATP was omitted from the incubation medium, a much greater proportion of the total

[1]Bacterial contamination could be reduced to relatively insignificant levels by autoclaving the water used to prepare all reagents, including the sucrose solutions for homogenization and washing (Dr. N. Gregson, personal communication).

Table 3

Effects of ribonuclease, chloramphenicol, and oligomycin on protein synthesis in mitochondria

Mitochondria were treated with ribonuclease as described in text. Chloramphenicol was added at 50 µg per ml and oligomycin at 3.0 µg per ml. Other conditions are described in Table 2.

Treatment	Specific Radioactivity, as c.p.m./mg protein (figures in parenthesis = % total radioactivity)			
	Mitochondria	Soluble Fraction	Membrane Fraction	Sediment Fraction
None	16.8	10.2(48)	42.9(25)	171(27)
+chloramphenicol	1.4	2.5	7.7	8.3
None (ATP omitted)	23.8	4.7(22)	14.5(8)	115(70)
+oligomycin (ATP omitted)	11.0	<1	1.2	78(>95)
None	46.2	11.8(24)	80.2(59)	390(17)
+ribonuclease	43.4	4.7(13)	24.8(21)	477(66)

radioactivity was recovered in the sediment fraction containing the bacteria. Under these conditions, the addition of oligomycin, which inhibits endogenous ATP synthesis in mitochondria, almost completely prevented labeling of the mitochondrial membrane fraction, but gave only relatively low inhibition in the fraction containing bacteria. Elsewhere it has been shown that maximum rates of protein synthesis in mitochondria require external ATP and a regenerating system; under these conditions oligomycin does not inhibit protein synthesis (8, 9). From these findings it is clear that added ATP is required for maximum rates of true mitochondrial protein synthesis; in its absence most of the amino acid incorporation observed is into bacterial protein.

In agreement with Roodyn et al. (1), treatment of mitochondria with ribonuclease was found to have only a slight effect on incorporation of leucine into unfractionated mitochondria. However, ribonuclease greatly reduced labeling of the membrane fraction, compensated by an increase in radioactivity recovered in the sediment fraction. Since it has been shown that ribonuclease greatly inhibits protein synthesis in mitochondria supported by external ATP (8), it may be concluded that the fraction of amino acid incorporation in mitochondrial preparations that is insensitive to ribonuclease is very largely of bacterial nature.

From the results reported here it may be concluded that true biosynthesis of mitochondrial protein may occur in vitro, that it requires added ATP, and that it may be

studied without interference from contaminating bacteria by following amino acid incorporation into the membrane fraction as described. Further details on these and other aspects of mitochondrial protein synthesis are described in another communication (8).

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